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3	2	"20010016951"	USPAT; US-PGPUB; EPO; DERWENT	2004/09/16 13:50
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TRANSGENIC ANIMAL MODEL FOR ALZHEIMER DISEASE

[0001] The present invention relates to an animal model useful for testing potential therapeutic agents for the treatment of neurodegenerative disorders, in particular Alzheimer's disease (AD).

[0002] More particularly the invention relates to an animal model involving transgenic manipulation of amyloid precursor protein (APP).

[0003] The lack of an experimental animal model for AD that reflects the pathological mechanisms is a major obstacle for both basic research and drug development. As one approach to such models, reproduction of characteristic lesions such as senile plaques, neurofibrillary pathology, and cell loss in certain areas of hippocampus and cortex can be attempted. However, it is presently unclear whether these lesions are cause or consequence of the disease process. An alternative approach for model generation is to use factors known to lead to the disease. Recently, genetic studies revealed mutations in APP, which cosegregate with early onset of familial AD in the fifth or sixth decade of life and follow an autosomal dominant inheritance pattern. Three distinct missense mutations affect codon 717 of APP (altering V717→I {hereinafter referred to as the London mutation}, V717→G and V717→F in the polypeptide), while codons 670/671 (altering K670→N and M671→L in the polypeptide, hereinafter referred to as the Swedish mutation) are altered in the APP gene of a Swedish AD pedigree (numbers according to APP770). These mutations flank the part of APP that gives rise to βA4, the principal component of the filaments deposited in plaques in the brains of AD patients. In vitro studies have indicated that the Swedish mutation leads to increased formation of a soluble form of βA4, while the APP717 mutations gives rise to a higher proportion of a longer βA4 variant which facilitates filament formation. Together with the finding that filamentous βA4 is toxic in vitro, this suggests that the APP mutations may lead to AD via a mechanism involving βA4, but other mechanisms cannot be excluded.

[0004] More recently, transgenic mice have been generated, expressing APP with mutations in codons 717 and 670/671, using several neuron-specific promotors to drive expression of human APP cDNAs. Although protein levels reaching or exceeding the amount of endogenous APP have been obtained, the full pattern of histological alterations characteristic of AD have not been seen in the transgenic mice.

[0005] It has now surprisingly been found that by appropriate selection of APP expression construct, high levels of transgene mRNA are obtained, which exceed the endogenous APP message by up to 10 fold, and result in correspondingly elevated protein levels. Moreover, on histological analysis, significant deposits of human βA4 peptide are observed. Additionally and even more importantly, hyperphosphorylation of the microtubule-associated protein tau is achieved, which is a pathological phenotype associated to AD. Furthermore, the deposits accumulate cholinesterase staining associated with a local distorsion of cholinergic fibers typically observed in AD. Both features have not been reported previously with analogous transgenic animals. The pathology is accompanied with selective neuron loss in distinct areas of the brain.

[0006] Accordingly in a first aspect the invention provides a recombinant DNA construct comprising a polynucleotide encoding a human APP polypeptide comprising the Swedish mutation, functionally linked to a Thy-1 promoter element, provided that the Thy-1 promoter element is a rodent, e.g. mouse, Thy-1 promoter element when the Swedish mutation is the only mutation present in the APP polypeptide.

[0007] Transgenic mice expressing said mutated human APP under control of said promotor have been found to develop a pathological phenotype which goes beyond that previously described by Games et al. [Nature 373, 523-527 (1995)], by combining APP and tau linked features of the AD pathology. Moreover, the mice have been found to present behavioural changes characteristic of AD, which has also never been reported before with transgenic animals.

[0008] It will be appreciated that such mice, by closely reflecting the AD pathology, as well as their transgenic cells, are particularly useful models of the disease.

[0009] Accordingly in a further aspect the invention provides transgenic non-human animals which exhibit both APP and tau-linked features, e.g. histological features, of AD pathology, and preferably also behavioural changes characteristic of AD.

[0010] Suitably the transgenic non-human animals express a human APP comprising the Swedish mutation or the Swedish mutation in combination with one or more additional mutations, in particular the London mutation. Suitably also the transgenic animal exhibits the features of AD pathology before 12 months of age preferably by about 6 months of age. Conveniently the transgenic animal is a rodent e.g. a mouse or a rat, preferably a mouse. This aspect of the invention includes transgenic cells derived from the transgenic non-human animal.

[0011] Without prejudice to the generality of the present invention, it appears that the level at which the transgene is expressed in the transgenic animal e.g. the level of transgene mRNA, is an important factor for obtaining AD pathology in the animal.

[0012] Thus in a further aspect the present invention provides a transgenic non-human animal cell, wherein DNA coding for a human APP having only one mutation is expressed at such a level that the amount of transgene mRNA exceeds the endogenous APP message by about 5 times, e.g. from 3 to 6 times, or more, e.g. from about 5 to about 10 times, as well as a transgenic non-human animal, e.g. a mouse or a rat, preferably a mouse, in the cells of which DNA coding for a human APP having only one mutation is expressed at such a level that the amount of transgene mRNA exceeds the endogenous message by about 5 times or more.

[0013] The only one mutation present in the APP polypeptide may comprise any APP mutation, including the Swedish mutation or the London mutation or other mutations at amino acid 717. Preferably the only one mutation is the Swedish mutation.

[0014] It furthermore appears that the number of genetic lesions influencing the production of βA4 introduced in a transgenic animal is another important factor for obtaining AD pathology in the animal.

[0015] The invention also provides a transgenic non-human animal cell, wherein DNA coding for a human APP having 2 mutations is expressed at such a level that the amount of transgene mRNA exceeds the endogenous APP message by about 2 times, e.g. from 1.5 to 3 times, as well as a transgenic non-human animal, e.g. a mouse or a rat, preferably a mouse, in the cells of which DNA coding for a human APP is expressed at such a level that the amount of transgene mRNA exceeds the endogenous message by about 2 times. Further the invention provides a transgenic non-human animal cell, wherein DNA coding for a human APP having 3 or more mutations is expressed at such a level that the amount of transgene mRNA exceeds the endogenous APP message by less than 2 times, e.g. from about 1 to 2 times, as well as a transgenic non-human animal, e.g. a mouse or a rat, preferably a mouse, in the cells of which DNA coding for a human APP is expressed at such a level that the amount of transgene mRNA exceeds the endogenous message by less than 2 times.

[0016] The 2 mutations or 3 or more mutations may comprise any combination of 2 or 3 or more APP mutations. Preferably, however, such multiple mutations comprise a combination of the Swedish and London mutations.

[0017] The DNA coding for human APP may comprise cDNA and/or genomic DNA, and is conveniently cDNA.

[0018] More particularly the present invention provides a transgenic non-human animal cell, wherein DNA encoding a human APP polypeptide comprising the Swedish mutation is expressed under the transcriptional control of a Thy-1 promotor element, as well as a transgenic non-human animal, e.g. a mouse or a rat, preferably a mouse, in the cells of which DNA encoding a human APP polypeptide comprising the Swedish mutation is expressed under the transcriptional control of a Thy-1 promotor element, provided that when the Swedish mutation is the only mutation present in the APP polypeptide the Thy-1 promoter element is a rodent, e.g. mouse, Thy-1 promoter element.

[0019] Transgenic animals according to the invention include animals into which the construct has been introduced directly as well as progeny of such animals which retain the ability to express the construct.

[0020] Cells manipulated according to the invention may be prepared by any known transfection technique. The DNA sequence may be introduced by direct genetic manipulation or into an earlier generation of the cell. Thus, the cells may be obtained from transgenic animals and cultured in vitro.

[0021] Also the transgenic animals may be generated according to well established methods, such as manipulation of embryos, e.g. by gene transfer into embryonic stem cells, retroviral infection of early embryos or pronuclear microinjection.

[0022] The pronuclear microinjection technique is preferred. Transcription units obtained from a recombinant DNA construct of the invention are injected into pronuclei of animal embryos and the obtained founder transgenics are bred.

[0023] The results obtained in the offspring can be analysed using various techniques well known in the art. Thus, for example, transgene APP mRNA expression is analysed by RNA blotting, the expression pattern of the transgene in the brain is determined by in situ hybridization, detection of APP in the brain is effected using immunoblotting techniques (western blot analysis) and the effects of the expression are studied by histology and inmunohistology.

[0024] Models based on cells and animals of the invention may be used for example to identify and assess the efficacy of potential therapeutic agents in neurodegenerative diseases, particularly in diseases where βA4 peptide is deposited and/or the microtubuleassociated protein tau is hyperphosphorylated, more particularly in AD. In particular such models may be used in screening or characterization assays for detecting agents likely to prevent βA4 deposit and/or hyperphosphorylation of tau.

[0025] Accordingly in a further aspect the invention comprises a method for testing a potential therapeutic agent for a specified condition, in particular a neurodegenerative disease, preferably AD, wherein a cell of the invention is used as target cell. More particularly it comprises such a method, wherein the agent is administered to a transgenic non-human animal of the invention. Moreover the invention comprises a screening or characterization assay consisting in or including such a method, as well as a screening assay kit comprising cells of the invention.

[0026] Methods for screening potential therapeutic agents using cell lines or animals are well known in the art. The cells and animals of the present invention may be used in analogous manner.

[0027] The recombinant cells may for example be incubated with the potential therapeutic agent and with antibodies recognizing βA4 amyloid in typical senile and diffuse plaques and/or with tau antibodies staining neurofibrillary tangles in the Alzheimer brain. In methods where the transgenic animals themselves are used, the effects of the potential therapeutic agent may be determined by carrying out various investigations on the animals after sacrifice. Also after administration of the potential therapeutic agent, the transgenic animal may undergo behavioural testing in order to monitor cognitive function.

[0028] The techniques of detection of βA4 and protein tau, including Western blot analysis, and the antibodies used therefor, are also well documented.

[0029] Compounds for use in the treatment of neurodegenerative diseases, which have been identified using an assay or assay kit as defined above, are also part of the present invention.

The following example illustrates the invention:

[0030] Expression Construct

[0031] Human APP751 cDNA carrying the Swedish double mutation is modified at the 5' end to reconstitute an optimal translation initiation sequence (GCC GCC ATG G).

[0032] This cDNA starting at above sequence and extending to nucleotide 3026 (Hind III site) is inserted into the Xho I cloning site of a pUC18-based vector containing an 8.1 kb EcoRI fragment comprising the mouse Thy-1.2 gene [Vidal et al. (1990) EMBO J. 9, 833-840]. The vector is modified such that a 1.5 kb BanI-XhoI fragment carrying exon 3 and flanking intervening sequences is replaced by a linker sequence encoding the unique Xho I recognition site [Mo-

As an example of construction of a cosmid vector for use in the instant invention, components which are assembled, in the 5' to 3' direction , include promoter and enhancer sequences of the prion protein gene, the coding region of an APP gene sequence of interest and transcriptional and translational termination sequences operably attached to a cosmid vector for delivery of the DNA constructs into the pronuclei of mouse eggs for expression of an APP gene in brain tissue. The enhancer sequences may include a 20 kb region upstream of the prion protein promoter and may also include the noncoding exon 1 and the 10 kb intron downstream of exon 1 from the prion protein gene or can include the coding sequence for more than one APP protein as described in, for example, WO92/11276. Using molecular genetic techniques well known in the art, the promoter/enhancer region of the prion protein gene may be isolated from a mammalian genomic cosmid clone used to create transgenic mice which express prion protein. The coding sequence of an APP gene is inserted between the promoter/enhancer region and the termination sequences at unique restriction site or sites such that the coding sequence is translated in-frame. An APP protein in transgenic brain tissue introduced using a cosmid vector as described above may be confirmed to be at least two to four-fold that of endogenous levels. A major obstacle to the creation of a transgenic model of AD has been the inability to overexpress transgenic APP protein in the brain of the transgenic animal. In some cases, mRNA is well expressed, but the protein is poorly expressed. This indicates that the strength of promoters used may be adequate, but that protein translation may not be optimal. Poor translation may result from a weak translation initiation sequence. Accordingly, it may be necessary to include a translation initiation sequence wherein the positions at minus three and plus four relative to the initiation codon are A and G, respectively. See Table 1 below.

TABLE 1

Transgene Translation Initiation Sequence Optimization

Transgene	Translation Initiation Sequence
	-3 +4
Hacos.CS0HuAPP695-V717Imyc	GCGATGCTG (SEQ ID NO:1) (native human APP)
Hacos.CS1	ACCATGCTG (SEQ ID NO:2)
Hacos.CS2	ACCATGGTG (SEQ ID NO:3)
Hacos.MoAPP695-WT	ACGATGCTG (SEQ ID NO:4) (native mouse APP)
Hacos.MoPrP-P101L	ATCATGGCG (SEQ ID NO:5) (native mouse PrP)

Any amyloid precursor protein sequence can be used to produce the transgenic animals of the invention. An APP protein sequence, as the term is used herein, means a sequence of the coding region of the APP gene which, when incorporated into the genome of the animal in multiple copies and expressed in the transgenic animal at supraendogenous levels, promotes a progressive neurologic disease in the transgenic animal. The neurologic disease is characterized by neurobehavioral disorder with gliosis and diminished glucose uptake and/or utilization in cortico-limbic brain structures. The coding sequence can be from a wild-type gene, or from a gene containing one or more mutations. The coding sequences can be a natural sequence or a synthetic sequence or a combination of natural and synthetic sequences. By mutant is intended any APP which has an amino acid sequence which differs from that of the native APP and includes substitutions, deletions, and the like. By wild-type APP is intended native APP as it occurs in the relevant host animal.

Native human APP is encoded by a single 400-kb gene comprised of 18 exons on chromosome 21. Alternative mRNA splicing gives rise to three APP isoforms. Two forms, APP751 and APP770 contain a Kunitz-protease inhibitor (KPI) region; the third, APP-695, lacks the KPI segment. Preferred sequences are those which are disease-linked. Examples of disease-linked mutations include a mutation at APP codon 693 (of APP770) linked to Dutch congophilic angiopathy (Levy, et al., (1990) *Science* 248:1124), a mutation in APP linked to familial AD, valine-isoleucine at codon 717 (of APP770) (Goate, et al., (1991) *Nature* 349:704–706), a mutation wherein the valine at codon 717 is replaced by phenylalanine or glycine (Chartier-Harlin, et al., (1991) *Nature* 353: 844–846; Murrell, et al., (1991) *Science* 254: 97–99); and in one family with both congophilic angiopathy and AD, a mutation wherein alanine is replaced by glycine at codon 692 (Hendriks, et al., (1992) *Nature Genetics* 1:218–221). In a Swedish kindred, a double mutation at codons 670 and 671, resulting in a substitution of the normal lysine-methionine dipeptide by asparagine-leucine was found (Mullan, et al., (1992) *Nature Genetics* 1:345–347). APP with K670N-M671L is reported to be associated with increased Aβ1–40 secretion (Citron et al. (1992) *Nature* 360: 672–674; Cai et al. (1993) *Science* 259: 514–516), while enhanced Aβ1–42 production is reported for APP with the V717I mutation (Cai et al. (1993), supra; Suzuki et al. (1994) *Science* 264: 1335–1340). To obtain animals with a progressive neurologic disease, while it can be used, it is unnecessary to use a coding sequence derived from an APP Gene with a mutation at the 717 locus; likewise, while it can be used, it also is unnecessary to use a coding sequence which includes a KPI region and/or splice sites within the coding region.

Table 2, below, lists some of the known amyloid precursor protein sequences, some of which are genetically linked to familial Alzheimer's disease.

TABLE 2[1]

Examples of APP Transgenes

Translation Initiation	APP ORF Species	ORF Size (Codons)	Mutation
CS1 or CS2	human, mouse or human/mouse chimeras	695 & 751 or 770	V717I V717G V717F VVM717/721/722IAV KM670/671NL770 A692G E693Q

[1]The abbreviations used in Table 2 refer to the following: CS1 = translation initiation sequence as represented in FIG. 6; CS2 = translation initiation sequence as represented in FIG. 7; V = valine; I = isoleucine; G = glycine; F = phenylalanine; M = methionine; A = alanine; K = lysine; N = asparagine; L = leucine; E = glutamate; Q = glutamine; ORF = open reading frame; numeral in the Mutation column refers to the mutated codon based upon the APP770 numbering system.

Of particular interest are novel chimeric APP genes, in which human Aβ sequences replace the Aβ region of mouse APP. A158,5 is a 4-kDA peptide derived from APP. Examination of human (Hu), mouse (Mo), and chimeric (Mo/Hu) APP processing in mouse cell lines indicates that tangible